Antibody analysis using the Protein 200 HT-2 Assay – A suitability test

Application Note

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Abstract

Protein formulation technology is an integral part of modern drug development and there is a growing need for sample throughput during formulation screening. The Protein 200 HT-2 assay, run on the Agilent 5100 Automated Lab-on-a-Chip Platform (ALP), can size and quantitate proteins within the 15 to 220 kDa size range. Analysis is performed under denaturing conditions in the absence or presence of a reducing agent. Protein samples are analyzed in an automated high throughput mode using a reusable microfluidic chip.

This Application Note describes the analysis of different antibody samples under reducing and non-reducing conditions. The linear dynamic range, the maximal and minimal loadable concentration, as well as sizing and quantification reproducibility for antibodies were determined. In addition, the robustness of the 5100 ALP was demonstrated by running a large sample set.

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Introduction

In recent years the number of protein-based pharmaceuticals reaching the marketplace has increased exponentially. However, the clinical application of these drugs is limited by a lack of desirable attributes for adequate absorption or distribution. It therefore becomes critical to formulate these drugs into a safe and stable environment and to choose efficacious delivery systems for the protein drug. Thus, protein formulation technology is an integral part of protein drug development. In order to find an optimal formulation for protein based therapeutics a formulation screen is usually carried out. This results in several hundreds of samples needing to be monitored for purity and stability and therefore increases the need for the industry to automate these analyses. This Application Note focuses on the analysis of antibody based drugs. Currently sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is still used to determine degradation and the by-products of antibodies. However, this method is very tedious, labor-intensive and difficult to automate. Additionally, size exclusion chromatography (SEC) is generally used to analyze antibody samples but due to very long run times is not very suitable as a screening method[1]. In order to overcome these disadvantages, the Agilent 5100 Automated Lab-on-a-Chip platform (ALP), in conjunction with the Protein 200 HT-2 assay, was investigated[2]. This technique should be used for the determination of purity of antibodies in the non-reduced and reduced mode for a high sample throughput instead of SEC and SDS-PAGE. The system was tested for its assay specification and robustness with respect to antibodies.

Material and methods

Three different antibody samples were used to perform the suitability test. Antibody 1, an IgG1 antibody was analyzed under non-reducing conditions only. Antibody 2, an IgG1 antibody, was analyzed under reducing conditions only. Antibody 3, an IgG4 antibody, was analyzed under both non-reducing and reducing conditions. Antibody 3 was used in a project with 10 subsequent 96 well plates in order to test the robustness of the system. The on-chip electrophoresis was performed on the Agilent 5100 ALP (part number G3002A: dual laser system) in combination with the Protein 200 HT-2 chip (part number G3011-87300) and the Protein 200 HT-2 reagent kit (part number G3011-68750). This chip can be used for up to 2000 samples and has 2 external capillaries that enable it to withdraw samples from a sealed well plate. The reagent kit includes sample buffer, protein standard ladder, storage buffer, and ready-to-use reagent plates. Unless otherwise noted (figure 7), samples were prepared according to the protocol included with the kit: 4 µL of each sample were combined using a multi-channel pipette with 2 µL of denaturing solution in a 96 well plate. For reducing conditions β-mercaptoethanol was added to the sample buffer of the kit as a reducing agent. For non-reducing conditions the reducing agent was substituted by deionized water. Sample plates were sealed with a Remp plate sealer (Easy Sealer, Remp AG Switzerland) and thin aluminum foil (part number G3009-41000) and heated for 5 minutes to 95 °C using a PCR machine with a heatable lid. The plates were then cooled to room temperature, the foil seal was removed, and 24 µL of deionized water was added to each well and mixed thoroughly by pipetting. The sample plates were then resealed with foil and inserted together with the Protein 200 HT-2 chip and a reagent plate into the depot of the Agilent 5100 ALP. After starting the run, the instrument performed the sample analysis automatically and unattended.

Data was exported from the Agilent 5100 expert HT software to Microsoft Excel for statistical analysis.

Results and discussion

For formulation testing on a large scale the linearity of antibody quantitation is very important.To test the Agilent 5100 ALP system for linearity of the non-reduced form, antibody 1 was used. Antibody samples were measured in the concentration range from 10-6000 µg/mL.The applied concentration was previously determined with SEC chromatography. Each concentration was analyzed twice in duplicate and only the concentration of the intact antibody peak was evaluated with the 5100 ALP.

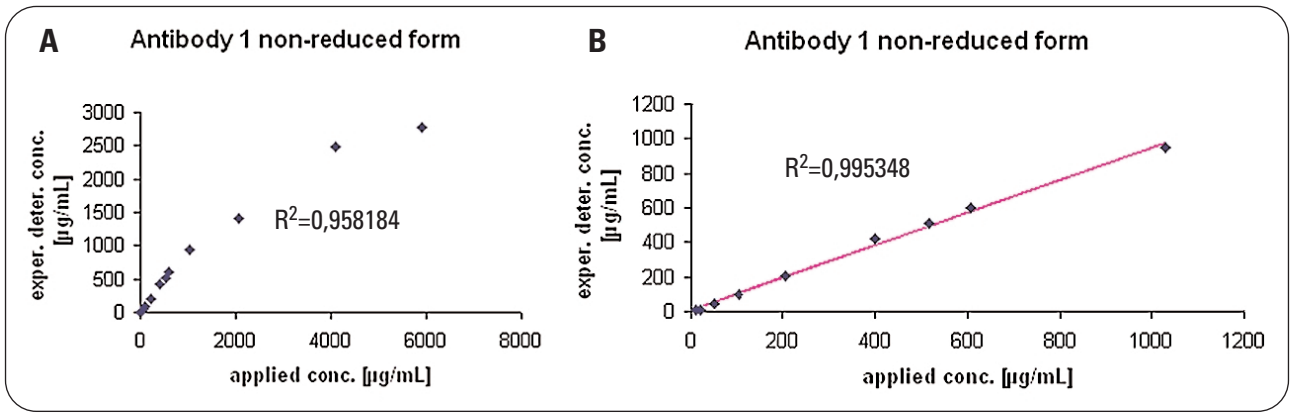

Figure 1
Linearity of the determined concentration with the Protein 200 HT-2 assay of antibody 1 in the non-reduced form as a function of the applied concentration. A) For the concentration range of 10-6000 µg/mL a linear regression coefficient of R^2 = 0.958184 was determined. B) For the concentration range of 10-1000 µg/ml a linear regression coefficient of R^2 = 0.995348 was determined. A good linearity could be achieved up to 1000 µg/mL.

Figure 1A shows a plot of determined concentration as a function of applied concentration for the whole concentration range of 10-6000 µg/mL. A linear regression coefficient R^2 = 0.958184 was determined. The criterion for a good linearity is a linear regression coefficient of at least R^2 = 0.98 which could only be reached up to 1000 µg/mL (figure 1B).
Figure 2 shows two example results of this data set as electropherograms. Figure 2A shows antibody 1 at a concentration of 1000 µg/mL, which is within the determined linear dynamic range, whereas figure 2B shows an electropherogram of antibody 1 at 6000 µg/mL, which is outside the linear dynamic and specified range. Although the high amount of sample applied did not disturb the analysis, the peak shape of the main peak (IgG1) clearly suffered and the resolution dropped significantly (see arrows in figure 2B). The linearity for the reduced form was determined using antibody 2 in the presence of β-mercaptoethanol as a reducing agent. The experimental setup was the same as for antibody 1. Figure 3A shows the plot of determined concentration as a function of applied concentration for the whole concentration range of 20-6000 µg/mL. This time the sum of the concentration of light chain and heavy chain was used for the concentration determination with the Agilent 5100 ALP. A linear regression coefficient R^2 = 0.973 was determined for the whole concen-

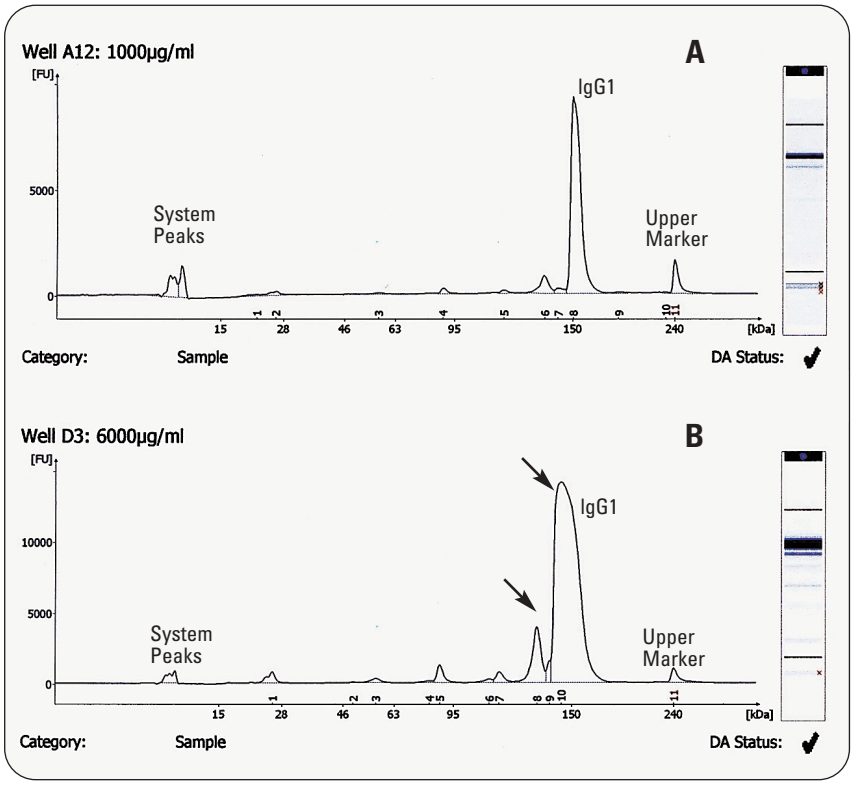

Figure 2
Example electropherograms of antibody 1 in the non-reduced form measured with the Protein 200 HT-2 assay A) Sample within the linear dynamic range (1000 µg/mL). B) Sample at too high concentration (6000 µg/mL). Overloading the system leads to abnormal peak shape and some loss of resolution (see arrows).

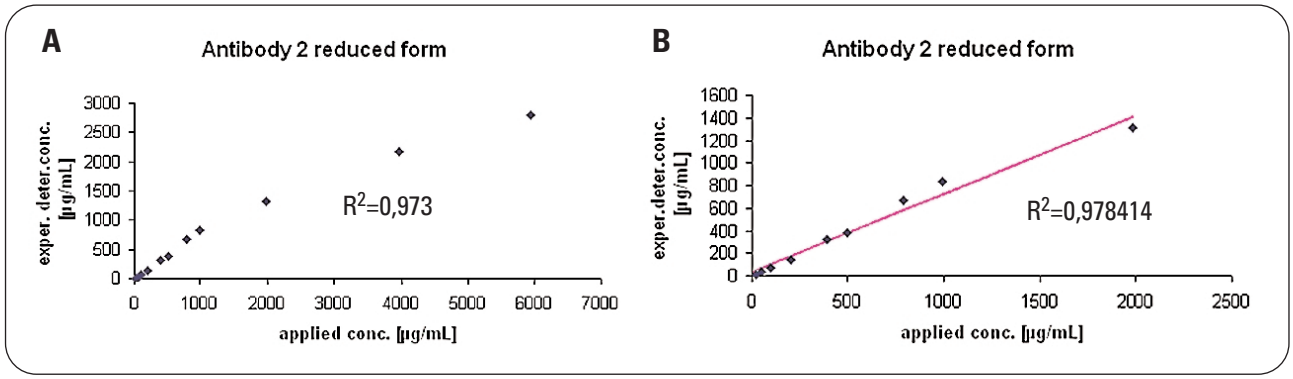

Figure 3
Linearity of the determined concentration with the Protein 200 HT-2 assay of antibody 2 in the reduced form as a function of the applied concentration. A) For the concentration range of 20-6000 µg/mL a linear regression coefficient of R^2 = 0.973 was determined. B) For the concentration range of 20-2000 µg/mL a linear regression coefficient of R^2 = 0.978 was determined. A good linearity could be achieved up to 2000 µg/mL.

tration range and R^2 = 0.978414 was determined for the concentration range of 20 – 2000 µg/mL. Therefore, a good linear dynamic range could be achieved up to 2000 µg/mL for the reduced form of antibody 2.

Similar to figure 2, figure 4 shows two example results of this data set as electropherograms. Figure 4A shows antibody 2 at a concentration of 2000 µg/mL, which is within the determined linear dynamic range, and figure 4B shows an overloaded sample of antibody 2 at 6000 µg/mL, which is outside the linear dynamic range. Again the high amount of sample applied did not disturb the analysis, however, the resolution dropped significantly (see arrow). The linear dynamic range is sufficient for antibody formulation screening purposes as 1 % of the main peak, which corresponds to 10 µg/mL for the non-reduced mode and 20 µg/mL for the reduced mode, is quantifiable.

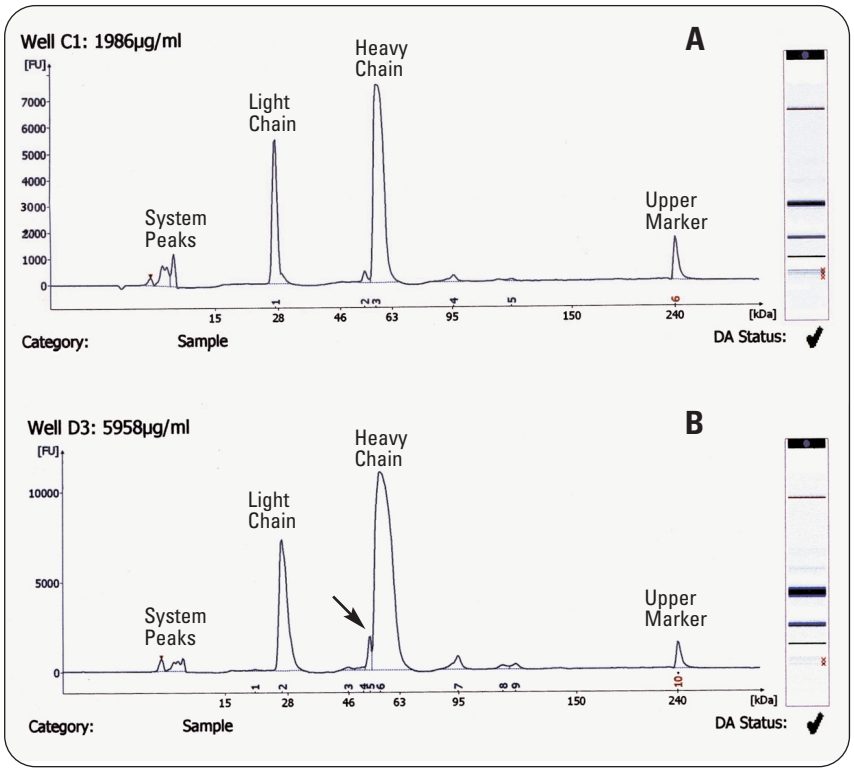

Figure 4
Example electropherograms of antibody 2 in the reduced form measured with the Protein 200 HT-2 assay. A) Sample within the linear dynamic range (2000 µg/mL). B) Sample at too high concentration (6000 µg/mL). Overloading the system leads to abnormal peak shape and loss of resolution (see arrow).

In addition to linearity, analyses of antibody 2 were also used to determine sizing reproducibility of the light chain and the heavy chain. The results are based on 15 independent analyses and are summarized in table 1.

	Theoretical molecular weight [kDa]	Determined average molecular weight [kDa]	CV [%]
Light chain	23.358	26.9	0.63
Heavy chain	49.237*	58.4	0.34

* Molecular weight including oligosaccharides (~50.8 kDa)

Table 1
Sizing reproducibility of antibody 2 in the reduced form: n = 15.

The theoretical molecular weight based on the amino acid sequence of the light chain is 23.358 kDa and of the heavy chain is 49.237 kDa. With the Protein 200 HT-2 assay, the average molecular weight determination revealed was 26.9 kDa for the light chain, with a sizing reproducibility of 0.63 %, and 58.4 kDa for the heavy chain, with a sizing reproducibility of 0.34 %. The slight absolute offset from the theoretical molecular weight is also known from other methods such as SDS-PAGE. This is due to the fact that each protein has its own individual migration behavior. The sizing reproducibility is excellent and even below the assay specification of 3 % CV [2].

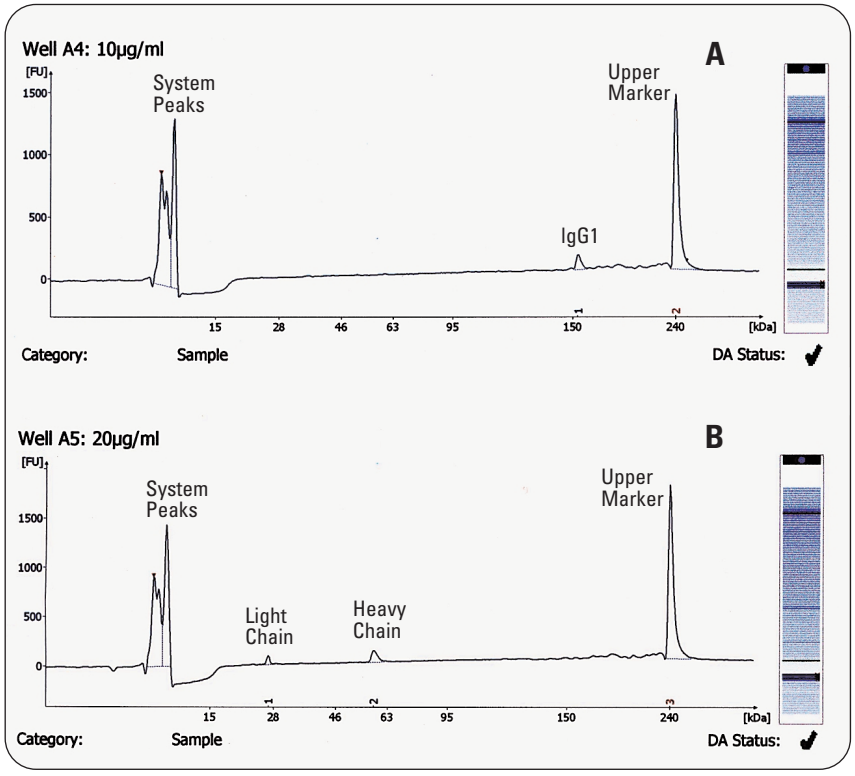

Figure 5
Example electropherograms at the limit of quantification (LOQ). A good integration by the Agilent 5100 expert HT software, as well as a signal to noise greater than 6, was used as criteria to determine the LOQ. A) 10 µg/mL of antibody 1 in the non-reduced form. B) 20 µg/mL of antibody 2 in the reduced form.

The limit of quantification (LOQ) was determined for both the non-reduced and the reduced mode of the antibodies. The LOQ's requirements were a good automated integration by the Agilent 5100 expert HT software and a signal to noise of at least 6. Figure 5 exemplifies typical results of the LOQ. Figure 5A shows antibody 1 in the non reduced form at 10 µg/mL, whilst figure 5B shows antibody 2 in the reduced form at 20 µg/mL.
In order to investigate the reliability and robustness of the system, a large sample set of 960 samples were analyzed in the same job. Therefore, five 96 well plates were each prepared twice with antibody 3 (with or without reducing agent).

The instrument records all sample data in digital form and transfers all information to a data base server making it available for long term storage, retrieval and data mining. Figure 6A shows the 10-plate overview of this job with the result flagging results as displayed by the software. The rules have been defined as follows:
The first rule searches for a peak of 143 kDa in a window of ± 3 % (corresponds to ± 4.29 kDa). If a peak is present in the sample, the software applies a color to the well dependent on the peak concentration. In this case a color gradient was defined where a low peak concentration corresponded to a light green color and a high peak concentration to a dark green color. The second rule was defined similarly but was designed to match the heavy chain of the reduced antibody. The rule searched for a peak of 56 kDa ± 5 % and applied a color gradient from light to dark red dependent on the peak concentration of the heavy chain. If both rules did not match then the well was marked light blue (default rule). As can be seen from the plate overview, 479 wells matched the rule for the intact antibody, 480 wells matched the rule for the heavy chain, and only one well was marked with the default rule. When handling large data sets this software feature is very convenient as it enables the operator to get a quick impression of the results. Similar result flagging rules could be applied for stability screening of antibody formulations. Figure 6B shows an electropherogram of antibody 3 at 1000 µg/mL. Since antibody 3 is an IgG4 antibody the half-antibody is visible in the non-reduced form, which is a typical behavior of IgG4 [3]. In the reduced form antibody 3 is

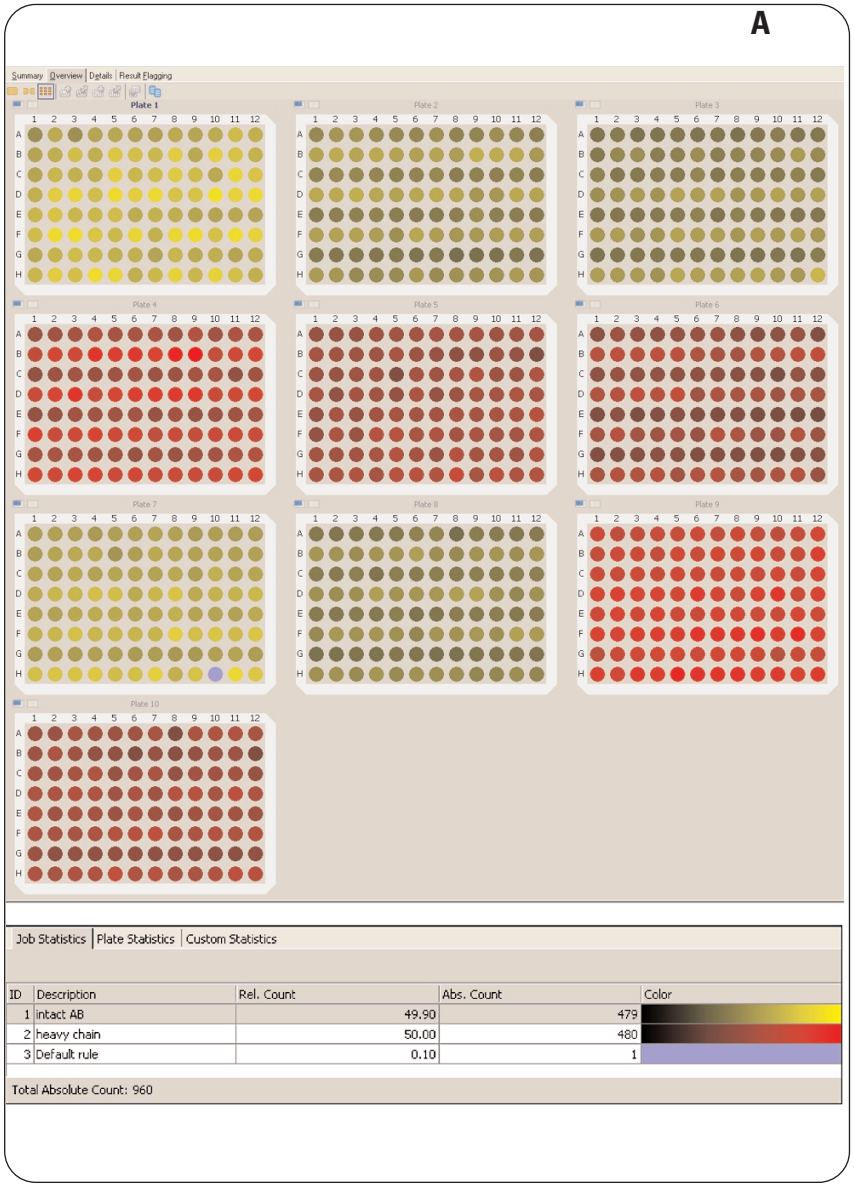

Figure 6
A ten 96 well plate job was performed with antibody 3 (IgG4), both in the reduced and non-reduced form, to test the robustness of the microfluidic system.
A) Plate overview with result flagging (details see text).

completely converted to the light and heavy chain (figure 6C). Easy visual sample comparison can also be done by using electropherogram overlays of different samples or by comparing virtual gel lanes in large gel-like images. For example, figure 6D depicts a gel-like image of 84 analyses of antibody 3 in the non-reduced form bracketed with Protein 200 HT-2 ladders (which are used for the alignment and size determination).

For quantification determination the upper marker is used as an internal standard[2]. The upper marker is included in the sample buffer of the kit and is added to the sample during sample preparation. Since very small volumes are used, namely only 4 µL of sample and 2 µL of denaturing solution (see material and methods), a test was done to see whether an upscaled sample preparation protocol would have a significant effect on the quantification reproducibility. Therefore, one 96-well-plate was prepared following the standard protocol and one 96 well plate was prepared with a protocol upscaled five times (figure 7). Both conditions were prepared manually with a multi-channel pipette. For the concentration determination the concentration of the main peak was determined. Interestingly, the upscaled protocol revealed no significant improvement over the standard protocol. With the standard protocol a quantification reproducibility of 6.7 % CV was determined and the upscaled protocol resulted in a quantification reproducibility of 5.5 % CV. However, this relatively small improvement does not justify upscaling the protocol since this would also result in a larger cost per analysis.

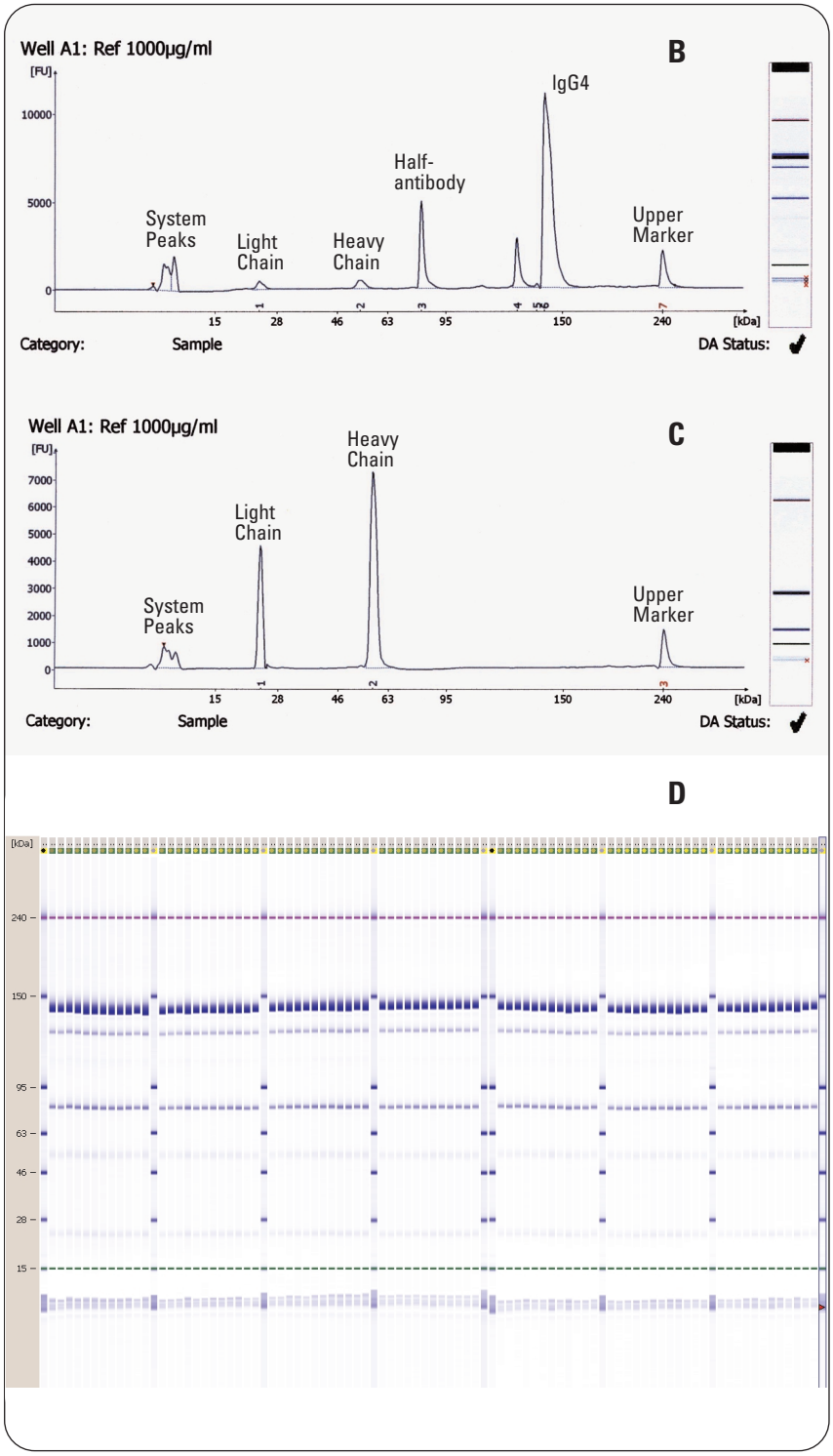

Figure 6
B) Example electropherogram of antibody 3 in the non-reduced form (1000 µg/mL).
C) Example electropherogram of antibody 3 in the reduced form (1000 µg/mL).
D) Gel-like image of 84 analysis of antibody 3 in the non-reduced form bracketed with Protein 200 HT-2 ladders.

Conclusions

In conjunction with the Agilent 5100 ALP instrument, the Protein 200 HT-2 assay provides a new tool for sizing and quantification of proteins in a high throughput mode. The data presented in this Application Note supports the suitability of this system as an antibody formulation screening method. Suitable linear dynamic range was determined to be 10 - 1000 µg/mL for the non reduced form of the tested antibody and 20 - 2000 µg/mL for the reduced form of the tested antibody. The system could be overloaded up to a concentration of 6000 µg/mL without any effect on the assay performance. The minimal loadable concentration (LOQ) was determined to be 10 µg/mL for the non-reduced form of the tested antibody and 20 µg/mL for the reduced form. Sizing reproducibility was below 1 % CV and quantification reproducibility was below 7 % CV. Upscaling of the sample preparation protocol had no major effect on the quantification reproducibility. Overall the system was very robust and no clogging or other interruptions were observed, even with large sample sets. In addition to these reliable results, the system has other significant advantages over SDS-PAGE and SEC in protein drug analysis. These advantages include drastically reduced time for results, fully unattended and automated operation, easy data evaluation, as well as convenient report and archiving tools.

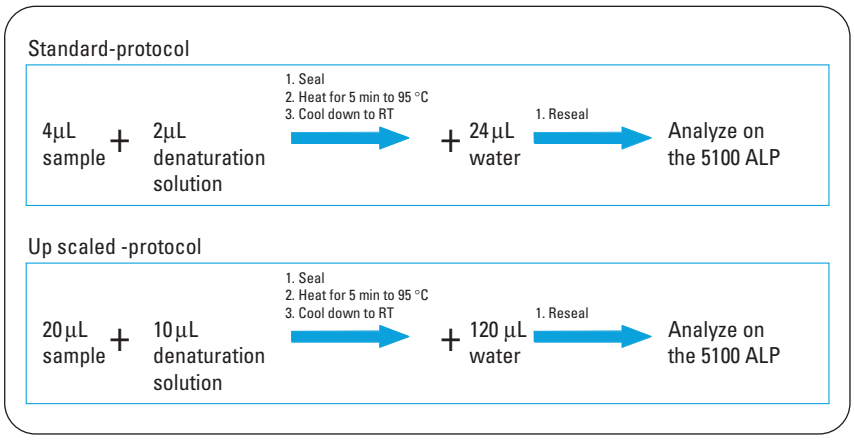

Figure 7
Schematic outline of the standard-protocol as well as an upscaled protocol for the protein sample preparation prior to the analysis of an 800 µg/mL sample: n = 96.

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